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2. Patent application number
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ZENECA Limited
15 Stanhope Gate
London, W1Y 6LN, GB

Patents ADP number *(if you know it)*

6254007002

[If] ~~the applicant~~ is a corporate body, give the country/state of its incorporation

4. Title of the invention

CHEMICAL COMPOUNDS

5. Name of your agent *(if you have one)*

GILES, Allen Frank

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ZENECA Pharmaceuticals
Intellectual Property Department
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CHEMICAL COMPOUNDS

This invention relates to polymorphisms in the human α_4 integrin subunit gene. The invention also relates to methods and materials for analysing allelic variation in the α_4 integrin subunit gene, and to the use of α_4 integrin subunit polymorphism in the diagnosis and treatment of integrin ligand mediated diseases such as multiple sclerosis, rheumatoid arthritis, atherosclerosis and allergic asthma.

The integrins are a family of heterodimeric cell surface receptors that are composed of noncovalently associated glycoprotein subunits (α and β) and are involved in the adhesion of cells to other cells or to extracellular matrix. The interactions between integrins and their protein ligands are fundamental for maintaining cell function, for example by tethering cells at a particular location, facilitating cell migration, or providing survival signals to cells from their environment. Ligands recognised by integrins include extracellular matrix proteins, such as collagen and fibronectin; plasma proteins, such as fibrinogen; and cell surface molecules, such as transmembrane proteins of the immunoglobulin superfamily and cell-bound complement. There are at least 14 different human integrin α subunits and at least 8 different β subunits and each β subunit can form a heterodimer with one or more α subunits. The specificity of the interaction between integrin and ligand is governed by the α and β subunit composition.

The α_4 integrin subunit comprises 999 amino acids and is formed from a 1038 amino acid precursor by the cleavage of a 39 amino acid N-terminal signal peptide. The core protein molecular weight is 111 kDa. There are 11 N-glycosylation sites in the extracellular region and the protein expressed on the cell surface usually has a molecular weight of 145 kDa, although it can also exist as a 180 kDa isoform. The 145 kDa form can be partially cleaved into 80 and 70 kDa fragments. The extracellular domain comprises amino acid residues 1-944, the transmembrane domain residues 945-967 and there is a short intracellular domain comprising residues 968-999. The N-terminal 432 amino acids contain seven sequence repeats which are thought to fold into a seven-bladed β-propeller. Ligands and a putative magnesium ion are predicted to bind to the upper face of the β-propeller while there are three calcium binding motifs on the lower face.

The α_4 subunit is known to form a heterodimer with either the β_1 or β_7 subunits. The integrin $\alpha_4\beta_1$, also known as Very Late Antigen-4 (VLA-4) or CD49d/CD29, is expressed on

numerous hematopoietic cells, including hematopoietic precursors, peripheral and cytotoxic T lymphocytes, B lymphocytes, monocytes, thymocytes and eosinophils, and established cell lines. $\alpha_4\beta_1$ has two main ligands, Vascular Cell Adhesion Molecule-1 (VCAM-1), also known as CD106, an immunoglobulin superfamily member expressed on the surface of activated vascular endothelial cells and a variety of other cells including dendritic cells, macrophages and fibroblasts, and an isoform of fibronectin containing the alternatively spliced type III connecting segment (CS-1 fibronectin). $\alpha_4\beta_7$ also recognises VCAM-1 and CS-1 fibronectin as ligands but will preferentially bind to Mucosal Addressin Cell Adhesion Molecule-1 (MAdCAM-1), another immunoglobulin superfamily member expressed on vascular endothelial cells, mainly in the small intestine and to a lesser extent the colon and spleen. $\alpha_4\beta_7$ is expressed on lymphocytes that preferentially home to gastrointestinal mucosa and gut-associated lymphoid tissue and may have a role in maintaining mucosal immunity.

The activation and extravasation of blood leukocytes plays a major role in the development and progression of inflammatory diseases. Cell adhesion to the vascular endothelium is required before cells migrate from the blood into inflamed tissue and is mediated by specific interactions between cell adhesion molecules on the surface of vascular endothelial cells and circulating leukocytes. α_4 integrins believed to have an important role in the recruitment of lymphocytes, monocytes and eosinophils during inflammation.

The affinity of leukocyte integrins for their ligands is normally low but activation of leukocytes increases integrin affinity. At sites of inflammation, leukocyte integrins are thought to be activated by chemokines which act via receptors on the leukocyte surface. Integrin affinity is thought to be regulated by conformational changes in the integrin subunits induced by intracellular signalling pathways acting on the integrin cytoplasmic tails.

Expression of α_4 integrin ligands is upregulated at sites of inflammation. VCAM-1 and MAdCAM-1 expression is upregulated on endothelial cells in vitro by inflammatory cytokines. VCAM-1 expression is upregulated in human inflammatory diseases such as rheumatoid arthritis, multiple sclerosis, allergic asthma and atherosclerosis while CS-1 fibronectin expression is upregulated in rheumatoid arthritis. MAdCAM-1 expression is upregulated in murine models of inflammatory bowel disease and insulin-dependent diabetes.

Monoclonal antibodies directed against the α_4 integrin subunit have been shown to be effective in a number of animal models of human inflammatory diseases including multiple

sclerosis, rheumatoid arthritis, allergic asthma, contact dermatitis, transplant rejection, insulin-dependent diabetes, inflammatory bowel disease, and glomerulonephritis.

$\alpha_4\beta_1$ /ligand binding has also been implicated in T-cell proliferation, B-cell localisation to germinal centres, haematopoietic progenitor cell localisation in the bone marrow, angiogenesis, placental development, muscle development and tumour cell metastasis.

Integrins recognise short peptide motifs in their ligands. The minimal α_4 integrin binding epitope in CS-1 is the tripeptide leucine-aspartic acid-valine (LDV) while VCAM-1 contains the similar sequence isoleucine-aspartic acid-serine (IDS). $\alpha_4\beta_7$ binds to a leucine-aspartic acid-threonine (LDT) motif in MAdCAM-1. Small molecule inhibitors of ligand binding to α_4 integrins have been designed based on these short peptide motifs. α_4 integrin antagonists, monoclonal antibodies directed at $\alpha 4$ integrins or their ligands and inhibitors of α_4 integrin ligand expression may have utility in the treatment of autoimmune, allergic and vascular inflammatory diseases, the prevention of tumour metastasis and in mobilisation of haematopoietic progenitor cells from bone marrow prior to tumour chemotherapy.

A cDNA encoding the α_4 integrin subunit has been cloned and published as a EMBL Accession number: L12002 (3567 bp) and all positions herein relate to the position therein unless stated otherwise or apparent from the context.

Szabo and McIntyre (1995), Molecular Immunology 32, 1543-54, disclosed a SNP in human integrin α_4 subunit at position 3061, which produces a Gln to Arg change in the subunit.

One approach is to use knowledge of polymorphisms to help identify patients most suited to therapy with particular pharmaceutical agents (this is often termed "pharmacogenetics") . Pharmacogenetics can also be used in pharmaceutical research to assist the drug selection process. Polymorphisms are used in mapping the human genome and to elucidate the genetic component of diseases. The reader is directed to the following references for background details on pharmacogenetics and other uses of polymorphism detection: Linder *et al.* (1997), Clinical Chemistry, **43**, 254; Marshall (1997), Nature Biotechnology, **15**, 1249; International Patent Application WO 97/40462, Spectra Biomedical; and Schafer *et al.* (1998), Nature Biotechnology, **16**, 33.

Clinical trials have shown that patient response to treatment with pharmaceuticals is often heterogeneous. Thus there is a need for improved approaches to pharmaceutical agent design and therapy.

The present invention is based on the discovery of five single nucleotide polymorphisms (SNPs) in the coding region of the human α_4 integrin subunit gene.

According to one aspect of the present invention there is provided a method for the diagnosis of a single nucleotide polymorphism in a α_4 integrin subunit in a human, which method comprises determining the sequence of the nucleic acid of the human at one or more of positions 740, 2273, 2446, 3311 and 3506 in the α_4 integrin subunit gene as defined by the positions in EMBL ACCESSION NO. L12002, and determining the status of the human by reference to polymorphism in the α_4 integrin subunit gene.

The term human includes both a human having or suspected of having a α_4 integrin subunit ligand mediated disease and an asymptomatic human who may be tested for predisposition or susceptibility to such disease. At each position the human may be homozygous for an allele or the human may be a heterozygote.

In one embodiment of the invention preferably the method for diagnosis described herein is one in which the single nucleotide polymorphism at position 740 is presence of C and/or T.

In another embodiment of the invention preferably the method for diagnosis described herein is one in which the single nucleotide polymorphism at position 2273 is presence of A and/or G.

In another embodiment of the invention preferably the method for diagnosis described herein is one in which the single nucleotide polymorphism at position 2446 is presence of C and/or T.

In another embodiment of the invention preferably the method for diagnosis described herein is one in which the single nucleotide polymorphism at position 3311 is presence of T and/or C.

In another embodiment of the invention preferably the method for diagnosis described herein is one in which the single nucleotide polymorphism at position 3506 is presence of C and/or T.

The method for diagnosis is preferably one in which the sequence is determined by a method selected from amplification refractory mutation system and restriction fragment length polymorphism.

In another aspect of the invention we provide a method for the diagnosis of α_4 integrin subunit ligand-mediated disease, which method comprises:

i) obtaining sample nucleic acid from an individual,

ii) detecting the presence or absence of a variant nucleotide at one or more of positions 740, 2273, 2446, 3311 and 3506 (as defined by the position in EMBL accession number L12002), in the α_4 integrin subunit gene and

iii) determining the status of the individual by reference to polymorphism in the α_4 integrin subunit gene.

Allelic variation at position 740 consists of a single base substitution from C (the published base), preferably to T. Allelic variation at position 2273 consists of a single base substitution from A (the published base), preferably to G. Allelic variation at position 2446 consists of a single base substitution from C (the published base), preferably to T. Allelic variation at position 3311 consists of a single base substitution from T (the published base), preferably to C. Allelic variation at position 3506 consists of a single base substitution from C (the published base), preferably to T. The status of the individual may be determined by reference to allelic variation at any one, two, three, four or all five positions optionally in combination with any other polymorphism in the gene that is (or becomes) known.

The test sample of nucleic acid is conveniently a sample of blood, bronchoalveolar lavage fluid, sputum, or other body fluid or tissue obtained from an individual. It will be appreciated that the test sample may equally be a nucleic acid sequence corresponding to the sequence in the test sample, that is to say that all or a part of the region in the sample nucleic acid may firstly be amplified using any convenient technique e.g. PCR, before analysis of allelic variation.

It will be apparent to the person skilled in the art that there are a large number of analytical procedures which may be used to detect the presence or absence of variant nucleotides at one or more polymorphic positions of the invention. In general, the detection of allelic variation requires a mutation discrimination technique, optionally an amplification reaction and optionally a signal generation system. Table 1 lists a number of mutation detection techniques, some based on the PCR. These may be used in combination with a number of signal generation systems, a selection of which is listed in Table 2. Further amplification techniques are listed in Table 3. Many current methods for the detection of allelic variation are reviewed by Nollau *et al.*, Clin. Chem. **43**, 1114-1120, 1997; and in standard textbooks, for example "Laboratory Protocols for Mutation Detection", Ed. by U. Landegren, Oxford University Press, 1996 and "PCR", 2nd Edition by Newton & Graham, BIOS Scientific Publishers Limited, 1997.

Abbreviations:

ALEX™	Amplification refractory mutation system linear extension
APEX	Arrayed primer extension
ARMS™	Amplification refractory mutation system
b-DNA	Branched DNA
CMC	Chemical mismatch cleavage
bp	base pair
COPS	Competitive oligonucleotide priming system
DGGE	Denaturing gradient gel electrophoresis
FRET	Fluorescence resonance energy transfer
LCR	Ligase chain reaction
MAdCAM-1	mucosal addressin cell adhesion molecule-1
MASDA	Multiple allele specific diagnostic assay
NASBA	Nucleic acid sequence based amplification
OLA	Oligonucleotide ligation assay
PCR	Polymerase chain reaction
PTT	Protein truncation test
RFLP	Restriction fragment length polymorphism
SDA	Strand displacement amplification
SNP	Single nucleotide polymorphism
SSCP	Single-strand conformation polymorphism analysis
SSR	Self sustained replication
TGGE	Temperature gradient gel electrophoresis
VCAM-1	Vascular Cell Adhesion Molecule-1
VLA-4	Very Late Antigen-4

<u>Table 1 - Mutation Detection Techniques</u>

General: DNA sequencing, Sequencing by hybridisation

Scanning: PTT*, SSCP, DGGE, TGGE, Cleavase, Heteroduplex analysis, CMC, Enzymatic mismatch cleavage

* Note: not useful for detection of promoter polymorphisms.

Hybridisation Based

Solid phase hybridisation: Dot blots, MASDA, Reverse dot blots, Oligonucleotide arrays (DNA Chips)

Solution phase hybridisation: Taqman™ - US-5210015 & US-5487972 (Hoffmann-La Roche), Molecular Beacons - Tyagi *et al* (1996), Nature Biotechnology, **14**, 303; WO 95/13399 (Public Health Inst., New York)

Extension Based: ARMS™, ALEX™ - European Patent No. EP 332435 B1 (Zeneca Limited), COPS - Gibbs *et al* (1989), Nucleic Acids Research, **17**, 2347.

Incorporation Based: Mini-sequencing, APEX

Restriction Enzyme Based: RFLP, Restriction site generating PCR

Ligation Based: OLA

Other: Invader assay

Table 2 - Signal Generation or Detection Systems

Fluorescence: FRET, Fluorescence quenching, Fluorescence polarisation - United Kingdom Patent No. 2228998 (Zeneca Limited)

Other: Chemiluminescence, Electrochemiluminescence, Raman, Radioactivity, Colorimetric, Hybridisation protection assay, Mass spectrometry

Table 3 - Further Amplification Methods

SSR, NASBA, LCR, SDA, b-DNA

Preferred mutation detection techniques include ARMS™, ALEX™, COPS, Taqman, Molecular Beacons, RFLP, and restriction site based PCR and FRET techniques.

Particularly preferred methods include ARMS™ and RFLP based methods. ARMS™ is an especially preferred method.

In a further aspect, the diagnostic methods of the invention are used to assess the efficacy of therapeutic compounds in the treatment of α_4 integrin subunit ligand mediated diseases such as autoimmune, allergic and vascular inflammatory diseases.

Assays, for example reporter-based assays, may be devised to detect whether one or more of the above polymorphisms affect transcription levels and/or message stability.

Individuals who carry particular allelic variants of the α_4 integrin subunit gene may therefore exhibit differences in their ability to regulate protein biosynthesis under different physiological conditions and may display altered abilities to react to different diseases. In addition, differences in protein regulation arising as a result of allelic variation may have a direct effect on the response of an individual to drug therapy. The diagnostic methods of the invention may be useful both to predict the clinical response to such agents and to determine therapeutic dose.

In a further aspect, the diagnostic methods of the invention, are used to assess the predisposition and/or susceptibility of an individual to diseases mediated by α_4 integrin

subunit ligands. This may be particularly relevant in the development of autoimmune, allergic and vascular inflammatory diseases and other diseases which are modulated by α_4 integrin subunit interactions. The present invention may be used to recognise individuals who are particularly at risk from developing these conditions.

In a further aspect, the diagnostic methods of the invention are used in the development of new drug therapies which selectively target one or more allelic variants of the α_4 integrin subunit gene. Identification of a link between a particular allelic variant and predisposition to disease development or response to drug therapy may have a significant impact on the design of new drugs. Drugs may be designed to regulate the biological activity of variants implicated in the disease process whilst minimising effects on other variants.

In a further diagnostic aspect of the invention the presence or absence of variant nucleotides is detected by reference to the loss or gain of, optionally engineered, sites recognised by restriction enzymes. In the accompanying Example 1 we provide details of convenient engineered restriction enzyme sites that are lost or gained as a result of a polymorphism of the invention.

According to another aspect of the present invention there is provided a nucleic acid comprising any one of the following polymorphisms:

the nucleic acid of EMBL ACCESSION No. L12002 with T at position 740 as defined by the position in EMBL ACCESSION No. L12002;

the nucleic acid of EMBL ACCESSION No. L12002 with G at position 2273 in the promoter sequence as defined by the position in EMBL ACCESSION No. L12002;

the nucleic acid of EMBL ACCESSION No. L12002 with T at position 2446 in the promoter sequence as defined by the position in EMBL ACCESSION No. L12002;

the nucleic acid of EMBL ACCESSION No. L12002 with C at position 3311 in the promoter sequence as defined by the position in EMBL ACCESSION No. L12002;

the nucleic acid of EMBL ACCESSION No. L12002 with T at position 3506 in the promoter sequence as defined by the position in EMBL ACCESSION No. L12002;

or a complementary strand thereof or a fragment thereof of at least 20 bases comprising at least one polymorphism.

Fragments are at least 17 bases, more preferably at least 20 bases, more preferably at least 30 bases.

The invention further provides nucleotide primers which can detect the polymorphisms of the invention.

According to another aspect of the present invention there is provided an allele specific primer capable of detecting a α_4 integrin subunit gene polymorphism at one or more of positions 740, 2273, 2446, 3311 and 3506 in the α_4 integrin subunit gene as defined by the positions in EMBL ACCESSION NO. L12002.

An allele specific primer is used, generally together with a constant primer, in an amplification reaction such as a PCR reaction, which provides the discrimination between alleles through selective amplification of one allele at a particular sequence position e.g. as used for ARMS™ assays. The allele specific primer is preferably 17- 50 nucleotides, more preferably about 17-35 nucleotides, more preferably about 17-30 nucleotides.

An allele specific primer preferably corresponds exactly with the allele to be detected but derivatives thereof are also contemplated wherein about 6-8 of the nucleotides at the 3' terminus correspond with the allele to be detected and wherein up to 10, such as up to 8, 6, 4, 2, or 1 of the remaining nucleotides may be varied without significantly affecting the properties of the primer.

Primers may be manufactured using any convenient method of synthesis. Examples of such methods may be found in standard textbooks, for example "Protocols for Oligonucleotides and Analogues; Synthesis and Properties," Methods in Molecular Biology Series; Volume 20; Ed. Sudhir Agrawal, Humana ISBN: 0-89603-247-7; 1993; 1st Edition. If required the primer(s) may be labelled to facilitate detection.

According to another aspect of the present invention there is provided an allele-specific oligonucleotide probe capable of detecting a α_4 integrin subunit gene polymorphism at one or more of positions 740, 2273, 2446, 3311 and 3506 in the α_4 integrin subunit gene as defined by the positions in EMBL ACCESSION NO. L12002.

The allele-specific oligonucleotide probe is preferably 17- 50 nucleotides, more preferably about 17-35 nucleotides, more preferably about 17-30 nucleotides.

The design of such probes will be apparent to the molecular biologist of ordinary skill. Such probes are of any convenient length such as up to 50 bases, up to 40 bases, more conveniently up to 30 bases in length, such as for example 8-25 or 8-15 bases in length. In general such probes will comprise base sequences entirely complementary to the corresponding wild type or variant locus in the gene. However, if required one or more

mismatches may be introduced, provided that the discriminatory power of the oligonucleotide probe is not unduly affected. The probes of the invention may carry one or more labels to facilitate detection.

According to another aspect of the present invention there is provided a diagnostic kit comprising an allele specific oligonucleotide probe of the invention and/or an allele-specific primer of the invention.

The diagnostic kits may comprise appropriate packaging and instructions for use in the methods of the invention. Such kits may further comprise appropriate buffer(s) and polymerase(s) such as thermostable polymerases, for example taq polymerase.

In another aspect of the invention, the single nucleotide polymorphisms of this invention may be used as genetic markers in linkage studies. This particularly applies to the polymorphisms at 2273 and/or 3311 (as defined by the position in EMBL ACCESSION NO. L12002) because of their relatively high frequency (see below). The α_4 integrin subunit gene has been mapped to chromosome 2q31-q32 (Fernandez-Ruiz *et al*, Europ. J. Immunol. **22**: 587-590, 1992).

According to another aspect of the present invention there is provided a method of treating a human in need of treatment with a α_4 integrin subunit ligand antagonist drug in which the method comprises:

i) diagnosis of a single nucleotide polymorphism in α_4 integrin subunit gene in the human, which diagnosis comprises determining the sequence of the nucleic acid at one or more of positions 740, 2273, 2446, 3311, and 3506 (as defined by the position in EMBL accession number L12002), and determining the status of the human by reference to polymorphism in the α_4 integrin subunit gene; and

ii) administering an effective amount of a α_4 integrin subunit ligand antagonist .

Preferably determination of the status of the human is clinically useful. Examples of clinical usefulness include deciding which antagonist drug or drugs to administer and/or in deciding on the effective amount of the drug or drugs.

α_4 integrin subunit ligand antagonist drugs have been disclosed in the following publications: international patent application WO 97/49731, Zeneca Limited; international patent application WO 97/02289, Zeneca Limited; international patent application WO 96/20216, Zeneca Limited; US patent 5510332, Texas Biotechnology; international patent application WO 96/01644, Athena Neurosciences; international patent application WO

96/01644, Athena Neurosciences and; international patent application WO 96/00581, Zeneca Limited. A α_4 integrin subunit ligand antagonist drug may act directly at α_4 integrin subunit heterodimer and/or at a ligand, such as VCAM, CS-1 fibronectin or MAdCAM-1 which binds to α_4 integrin subunit heterodimers, $\alpha_4\beta_1$ or $\alpha_4\beta_7$. VLA-4 antagonists as anti-inflammatory agents have been reviewed by Lin KC & Castro AC in Curr. Opin. Chem. Biol. (1998), **2**: 453-457.

According to another aspect of the present invention there is provided use of a α_4 integrin subunit ligand antagonist drug in preparation of a medicament for treating a α_4 integrin subunit ligand mediated disease in a human diagnosed as having a single nucleotide polymorphism at one or more of positions 740, 2273, 2446, 3311, and 3506 (as defined by the position in EMBL accession number L12002).

According to another aspect of the present invention there is provided a pharmaceutical pack comprising α_4 integrin subunit ligand antagonist drug and instructions for administration of the drug to humans diagnostically tested for a single nucleotide polymorphism at one or more of positions 740, 2273, 2446, 3311, and 3506 (as defined by the position in EMBL accession number L12002).

The invention will now be illustrated but not limited by reference to the following Examples. All temperatures are in degrees Celsius.

In the Examples below, unless otherwise stated, the following methodology and materials have been applied.

AMPLITAQ™ ,available from Perkin-Elmer Cetus, is used as the source of thermostable DNA polymerase.

General molecular biology procedures can be followed from any of the methods described in "Molecular Cloning - A Laboratory Manual" Second Edition, Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory, 1989).

Electropherograms were obtained in a standard manner: data was collected by ABI377 data collection software and the wave form generated by ABI Prism sequencing analysis (2.1.2).

Example 1

Identification of Polymorphisms

1. Methods

c-DNA Preparation

RNA was prepared from lymphoblastoid cell lines from Caucasian donors using standard laboratory protocols (Chomczynski and Sacchi, Anal. Biochem. **162**, 156-159, 1987) and used to generate first strand cDNA (Gubler and Hoffman, Gene **25**, 263-269, 1983).

Template Preparation

Templates were prepared by PCR using the oligonucleotide primers and annealing temperatures set out below. The extension temperature was 72° and denaturation temperature 94°; each step was 1 minute. Generally 100 pg cDNA was used in each reaction and subjected to 40 cycles of PCR.

Fragment	**Forward Oligo**	**Reverse Oligo**	**Annealing Temp**	**$MgCl_2$**	**DMSO**
290-811	290-310	790-811	64°	1.0 mM	5 %
2119-2630	2119-2139	2609-2630	64°	1.5 mM	0
2961-3550	2961-2982	3528-3550	60°	1.0 mM	0

For dye-primer sequencing the forward primers were modified to include M13 forward sequence (ABI protocol P/N 402114, Applied Biosystems) at the 5' end of the oligonucleotides.

Dye Primer Sequencing

Dye-primer sequencing using M13 forward and reverse primers was as described in the ABI protocol P/N 402114 for the ABI Prism™ dye primer cycle sequencing core kit with "AmpliTaq FS"™ DNA polymerase, modified in that the annealing temperature was 45° and DMSO was added to the cycle sequencing mix to a final concentration of 5 %.

The extension reactions for each base were pooled, ethanol/sodium acetate precipitated, washed and resuspended in formamide loading buffer.

4.25 % Acrylamide gels were run on an automated sequencer (ABI 377, Applied Biosystems).

2. Results

Novel Polymorphisms

Integrin Alpha-4 cDNA
EMBL Accession No L12002
ID HSITGA4
Ref Takada *et al*, EMBO J. **8**: 1361-1368, 1989

Position	Published	Variant	Amino acid change	RFLP	Frequency
740	C	T		NO	1/52
2273	A	G		eng'+Acl I	32/54
2446	C	T	thr-met	eng'+Bsp HI	1/54
3311	T	C		eng'-Sph I	29/54
3506	C	T		eng'+Spe I	2/52

Frequency is the allele frequency of the variant allele in control subjects.

Example 2

Engineered restriction site for detection of polymorphisms

Standard methodology can be used to detect the polymorphism at positions 2273, 2446, 3311 and 3506 (as defined by the position in EMBL ACCESSION NO. L12002) based on the materials set out below using a cDNA template.

Position	Diagnostic Fragment	Forward primer	Reverse primer
2273	2119-2297	2119-2139	2274-2297 Acl I
2446	2422-2630	2422-2445 Bsp HI	2609-2630
3311	2961-3335	2961-2982	3312-3335 Sph I
3506	3481-3564	3481-3505 Spe I	3542-3564

Primer Sequences

2274-2297 Acl I	GGCACAAAACCTTGCAAAGTTTAA
2422-2445 Bsp HI	ATGCTGGAGATGATGCATATGTCA
3312-3335 Sph I	ATGATGTAGTCCTTCCAGTAGAGC
3481-3505 Spe I	GAAGAGACAGTTGGAGTTATATCAC

G at position 2273 creates an Acl I site in the diagnostic fragment, 2119-2297, described above. T at position 2446 creates a Bsp HI site in the diagnostic fragment, 2422-2630, described above. T at position 3311 creates a Ssp I site in the diagnostic fragment, 2961-3335, described above. T at position 3506 creates a Spe I site in the diagnostic fragment, 3481-3564, described above.